Anaerobe 27 (2014) 100–105

Contents lists available at ScienceDirect

Anaerobe

journal homepage: www.elsevier.com/locate/anaerobe

Pathogenesis and toxins

# Relationship between gastrointestinal dysbiosis and *Clostridium botulinum* in dairy cows

Monika Krüger [a], Awad A. Shehata [*,a,b], Anke Grosse-Herrenthey [a], Norman Ständer [a], Wieland Schrödl [a]

[a] Institute of Bacteriology and Mycology, Faculty of Veterinary Medicine, Leipzig University, An den Tierkliniken 29, 04103 Leipzig, Germany
[b] Avian and Rabbit Diseases Department, Faculty of Veterinary Medicine, Sadat City University, Egypt

ARTICLE INFO

*Article history:*
Received 23 December 2013
Received in revised form
25 March 2014
Accepted 27 March 2014
Available online 16 April 2014

*Keywords:*
*C. botulinum*
Dysbiosis
Gut microbiota
BoNT
Protozoa

ABSTRACT

The gastrointestinal tract is a balanced ecosystem that can get out of balance and predisposed to clostridial diseases or other pathological conditions. The objective of the present study was to evaluate the gut microbiota in dairy cows suffering from chronic botulism. Cows were investigated for *Clostridium* (*C.*) *botulinum* in faeces and rumen fluids. In order to study the relationship between botulism and gastrointestinal microbiota, faeces and rumen fluid were tested for bacterial composition using conventional microbiological culture techniques and fluorescence in situ hybridization (FISH). Protozoa were analyzed in rumen fluid microscopically. The presence of *C. botulinum* was associated with specific changes in the faecal microbiota, especially a significant reduction of total aerobic bacteria, total anaerobic bacteria, enterococci, *Clostridium perfringens* and yeast and fungi. Also *C. botulinum* positive rumen fluid had significantly more *Bacteroides* spp., *C. histolyticum* group, Alfa- proteobacteria, Gammaproteobacteria, and sulfate-reducing bacteria; as well as significantly fewer *Euryaracheota*, and the protozoa *Epidinium* spp. *Dasytricha* spp., *Diplodiniinae* spp. and *Ophryoscolex* spp. In conclusion, *C. botulinum* is common in dairy cows in Germany but the incidence of botulism is associated with microbial changes and composition in the gastrointestinal tract. Bacteria, yeast and protozoa appear to be crucial in the colonization process; however, the chronology of these events and role of each microbial group needs further evaluation.



## 1. Introduction

The bacterium *Clostridium botulinum* is widespread in the environment. It can be found in soil, dust, manure, slaughter-house wastes, and residue of biogas plants, bio-compost and mud. The gastrointestinal tract of humans and animals is also a habitat for this agent. So far, seven serologically different botulinum neurotoxin (BoNT) types (A–G) are known that block the release of acetylcholine at the neuromuscular junctions. The BoNT may be taken up orally if preformed in food or feed (intoxication) [1–5]. In addition to the feed-borne diseases in domestic animals, another form of botulism is reported in cattle, called “visceral botulism” or “chronic botulism”. This form of botulism is thought to be caused by colonization of the lower intestine with *C. botulinum* and the subsequent production of BoNTs [2,5]. The presumed cause of clostridial colonization in the intestinal tract is microbial imbalance in the digestive tract accompanied by replication and toxin production as part of a multifactorial disease.

Gut microbiota have continuous communication with host cells and form long-lasting, interactive associations with their host. These associations play a critical role in conservation of mucosal immune function, epithelial barrier integrity, motility, and nutrient absorption [6–8]. Under normal conditions, gut microbiota display a symbiotic relationship with the host to contribute to its intestinal health; however, a disturbance in normal microbiota of the intestinal tract can lead to an imbalance of host–microbe relationships, known as “dysbiosis” [9]. Disturbances within this system by feed, heavy metals, toxic substances, bacterial toxins antibiotics, etc. can lead to localized inflammation, extensive infection or intoxication [10–12]. Protozoa are an important part of the rumen microbiota. They serve to reduce bacterial populations in the rumen and any changes in rumen bacteria influence their population [13]. Rumen protozoa represent an appreciable proportion of the rumen biomass [14] and have much longer generation time than bacteria (8–36 h vs. about 20 min). They depend on rumen bacterial support for vitamin B complex and vitamin K.

* Corresponding author. Institute of Bacteriology and Mycology, Faculty of Veterinary Medicine, Leipzig University, An den Tierkliniken 29, 04103 Leipzig, Germany. Tel.: +49 03419738183; fax: +49 03419738199.
*E-mail address:* shehata@vetmed.uni-leipzig.de (A.A. Shehata).

http://dx.doi.org/10.1016/j.anaerobe.2014.03.013
1075-9964/

Little is known about the role of immunological homeostasis in the gastrointestinal tract of cattle. It is postulated that under dysbiotic conditions, facultative pathogenic bacteria are able to induce disease because otherwise antagonistic bacterial populations fail to suppress them [15]. Thus, we hypothesize that colonisation and neurotoxin generation by *C. botulinum* in the gastrointestinal tract of cows also requires this precondition. In the last 15 years, *C. botulinum* associated chronic diseases have increased in Germany without known causes. The objective of this study was to shed light on the distribution of *C. botulinum* in dairy cows and its relationship to the microbial (bacteria, protozoa) composition of gut and rumen fluid.

## 2. Materials and methods

### 2.1. Animals

Cows at 16 farms, located in Saxony, Thuringia, Saxony–Anhalt and Schleswig Holstein, Germany were investigated to update the epidemiological situation of *C. botulinum* during the period 2008–2010. Cows of fourteen farms showed clinical signs of botulism while cows in 2 farms (designated GZ and K) were apparently healthy.

### 2.2. Samples

A total of 484 faecal specimens collected from the 16 farms were tested for *C. botulinum*. Faecal specimens (about 100 g per animal) were taken from *Ampulla recti*. All specimens were collected within three to 14 days after giving birth. Five farms (GH, R and E were diseased and had the highest percentages of botulinum positive cows; GZ and K were apparently healthy and had the lowest detection rate of botulinum positive cows) from the 16 farms were selected for further investigation (Table 1). Rumen fluids, taken orally using a pumped stomach tube, were tested for protozoa and *C. botulinum*. Faeces and rumen fluid samples from the selected 5 farms were further investigated by conventional microbiological culture techniques and fluorescence in situ hybridization (FISH), respectively.

### 2.3. Indirect detection of C. botulinum

The presence of the BoNT upon incubation of faecal and rumen specimens was considered predictive of the presence of the bacterium *C. botulinum*. Faecal specimens and rumen fluids were tested for *C. botulinum* by diluting samples 1:10 (0.5 g or 0.5 ml with 4.5 mL) in reinforced clostridial medium (RCM; SIFIN, Berlin, Germany), vigorously mixing, heating at 80 °C for 10 min and incubating at 37 °C for 7 d in anaerobic chamber (MACS anaerobic Workstation, Don Whitley Scientific Limited, West Yorkshire, England) before subsequent storage at −25 °C until analysed. After thawing, the culture samples were centrifuged at 7000 × g for 15 min and the clear supernatant was analysed for type specific BoNTs/A–E with polyclonal antibodies using ELISA [16]. The relative units (RUs) of BoNTs were calculated with the measured optical densities (OD) values as follow: (sample OD minus twice the value of the control OD [BoNT – negative sample of bovine faeces]) multiplied by dilution factors [17].

### 2.4. Bacterial enumeration in faeces and rumen fluid

#### 2.4.1. Bacterial cultivation

A total of 237 faecal samples collected from the selected 5 farms (GH, R, E, GZ, and K) were investigated by conventional microbiological culture techniques. Faecal specimens (0.5 g in 4.5 mL PBS) were serially diluted in PBS for quantitative bacterial investigations. Dilutions were tested for total aerobic cell numbers developing on sheep blood agar (Oxoid, Germany), Gram negative cell numbers on Gassner agar (SIFIN, Berlin), enterococci on *Citrate azide* tween carbonate agar (CATC agar, SIFIN, Berlin), total anaerobe cell numbers on sheep blood agar (OXOID, Germany), *Lactobacilli* on deMan, Rogosa and Sharpe Lactobacillus agar (MRSA agar, SIFIN, Berlin), *Bacteroides* spp., on sheep blood agar supplemented with vitamin K, *Clostridium perfringens* on sheep blood agar containing polymyxin B and neomycin, and *yeasts* and fungi on Sabouraud agar (SIFIN, Berlin). The total aerobic cell numbers, Gram negative cell numbers and enterococci were cultured aerobically at 37 °C for 24 h. The total anaerobic cell numbers, *Lactobacilli* and *Bacteriodes* were cultured at 37 °C for 48 h in anaerobic chamber. To differentiate the total anaerobic cells number from aerobic cells, samples were cultivated on sheep blood agar in which one plate is cultivated aerobically and the second is incubated anaerobically. *Yeasts* and fungi were cultured aerobically at 37 °C for 5 days.

#### 2.4.2. Fluorescence in situ hybridization (FISH) of rumen fluid

A total of 182 rumen fluid samples were investigated using FISH test. Briefly, samples were fixed in ice-cold ethanol (1:1, v/v), methanol (1:1, v/v) and fresh paraformaldehyde (1:3, v/v) as described previously [18,19] and hybridized using fluorophore (indocarbocyanin Cy3) labelled 16S/23S rRNA-targeted oligonucleotide probes on silanized microscope slides [20]. The following probes were used in this study: bacteria (Eub338) [21], *Archea*

**Table 1**
*Clostridium botulinum* types in faeces and rumen fluid of cows at five German dairy farms.

| Farm | Animal number | Health status | Sampling | | *C. botulinum* | *C. botulinum* faeces | | | | | *C. botulinum* in ruminal fluid | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Faeces | Rumen fluid | | A | B | C | D | E | A | B | C | D | E |
| GH | 700 | Diseased | 59 | 25 | Positive | 1 | 0 | 2 | 0 | 0 | 2 | 0 | 1 | 0 | 0 |
| | | | | | Suspected | 4 | 0 | 2 | 0 | 0 | 5 | 0 | 0 | 0 | 0 |
| R | 380 | Diseased | 89 | 88 | Positive | 6 | 0 | 1 | 0 | 0 | 13 | 2 | 1 | 0 | 0 |
| | | | | | Suspected | 12 | 0 | 5 | 0 | 0 | 22 | 1 | 0 | 1 | 0 |
| E | 550 | Diseased | 25 | 57 | Positive | 0 | 0 | 0 | 0 | 1 | 1 | 1 | 1 | 2 | 0 |
| | | | | | Suspected | 3 | 0 | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 |
| GZ | 652 | Healthy | 30 | *30* | Positive | 0 | 0 | 0 | 0 | 23 | 0 | 0 | 0 | 0 | 0 |
| | | | | | Suspected | 7 | 0 | 1 | 0 | 2 | 0 | 0 | 1 | 0 | 0 |
| K | 280 | Healthy | 35 | 25 | Positive | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | | | | Suspected | 7 | 0 | 2 | 0 | 0 | 5 | 0 | 0 | 0 | 0 |
| Total | | | 238 | 225 | Positive | 7 (2.9% | 0 | 4 (1.6%) | 0 | 24 (10.1%) | 16 (7.1%) | 3 (1.3%) | *3 (1.3%)* | *2 (0.8%)* | 0 |
| | | | | | Suspected | 33 (13.9%) | 0 | 10 4.2% | 0 | 3 (1.2%) | 32 (14.2%) | 1 (0.4%) | 1 (0.4%) | 1 (0.4%) | 0 |

Positive (BoNT concentration in enrichment culture of faeces or rumen fluids ≥ 10 RU/g or mL).
Suspected (BoNT concentration in enrichment culture of faeces or rumen fluids < 10 RUg or mL).